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Molecular Recognition in Antibody/Antigen Complexes N00014-89-J-3052

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The objectives of this project were to characterize the structural features and intermolecular forces governing affinity and selectivity in an antibody-antigen complex of known structure, and to improve methods of simulation of protein-protein interactions. The antilysozyme antibody HyHEL-10, for which the structure of the complex with antigen is known by crystallography, was used as the primary model system. Theory and modeling efforts have included: analysis of the effects of mutations on the free energy of HyHEL-10/lysozyme binding; prediction of the conformations of antibody hypervariable loops based on primary sequence data; and fundamental studies of biomolecular electrostatic interactions in solution, including the development of tools for modeling electrostatically-mediated diffusional encounter. Experimental efforts have focused on development of recombinant expression systems for the lysozyme antigen and for the Fab fragment of the HyHEL-10 antibody, for mutagenic perturbation of key intermolecular contacts and development of biophysical methods of determining thermodynamic driving forces and effects of mutation on affinity and specificity.

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OBJECTIVE: To characterize the structural features and intermolecular forces governing affinity and selectivity in antibody-antigen complexes of known structure, and to improve methods of simulation of protein-protein interactions.

ACCOMPLISHMENTS: In the Theory and Modeling area, this project has made progress on three different subprojects: analysis of the effects of mutations on the free energy of binding of lysozyme and the antibody HyHEL-10; prediction of the conformation of antibody hypervariable loops based on primary sequence data; and fundamental studies of biomolecular electrostatic interactions in solution, including the development of generally-applicable tools for modeling electrostatically-mediated diffusional encounter.

Molecular dynamics simulations and the thermodynamic cycle-perturbation method are used in the first subproject to predict the effects of substituting Asp for the wild type Asn31L in the antibody HyHEL-10. Because the Asn residue forms a hydrogen bond with Lys 96 of the lysozyme antigen, the substitution may result in increased affinity. A careful analysis of this possibility requires an examination of the adequacy of the model potential function and the possibility that different rotational isomeric states of the sidechains may contribute to the overall thermodynamics of binding, so recent work has addressed these issues. The CHARMM potential function was found to contain a questionable (three-fold) torsional potential for the chi 2 dihedral angle of Asn and Asp, and more appropriate potentials have been explored. The free energy cost (potential of mean force) for rotating chi 1 and chi 2 of these sidechains has been evaluated by molecular dynamics for models of the free antibody and the antibody-antigen complex.

We have shown that the potential functions lead to good agreement with experimental data for the relative free energies of hydration of the Asp and Asn residues, and the thermodynamic cycle perturbation method has been used to predict the thermodynamic effects of the mutation. Although such calculations are still extremely challenging if they are done carefully, the current results make clear that this method will become an important tool for molecular design as the performance of computers continues to increase. This work will be submitted for publication shortly as a joint paper (McCammon, Pomes, Willson).

Antibody Structure Prediction. The first paper submitted on this subject described the use of knowledge-based methods to predict conformations of the hypervariable loops of the anti-insulin antibody, 125. In the second paper, (J. J. Tanner et al., Biopolymers 32, 23 (1992)), we have shown how molecular dynamics refinement of an explicitly-hydrated model can be used to improve structure predictions based on

sequence data and homology arguments. Homology-based structures inevitably contain errors in dihedral angles, etc. By placing such a structure in a box of water and then carrying out careful equilibration and simulation of the antibody and surrounding solvent, local stresses in the structure can be relaxed in a realistic environment. An X-ray diffraction test of this structure prediction is in progress.

Electrostatic Interactions and Diffusional Encounters. Once one knows the structure of an antibody (by model-building or by experiment), the next questions one might ask are (1) what are the long-range forces that might govern the approach of an antigen, and (2) what is the actual dynamics of the encounter? Our UHBD program for calculating electrostatic fields around proteins, and for simulating the diffusional encounters of molecules subject to such fields, has been the subject of widespread interest, as described in several papers previously submitted. In one paper (M. E. Davis and J. A. McCammon, J. Comp. Chem. 12, 909 (1991)), we have introduced an improved method for calculating the electrostatic force field around a protein using the Poisson-Boltzmann equation. The improvement involves the smoothing of the dielectric boundary between the protein and the solvent; this smoothing leads to significantly more accurate results near the protein surface, where the final steps in binding occur. In a second paper (M. E. Davis, et al., Meth. Enzymol., 202, 473 (1991)), we described how Brownian dynamics simulations can be used to generate representative trajectories and evaluate rate constants for protein-ligand binding. In work that is now in progress (McCammon, Subramaniam, Willson), these techniques are being used to study the binding of lysozyme to anti-lysozyme antibodies.

In the Experimental area, efforts have focused on construction of recombinant expression systems for the lysozyme antigen and for the Fab fragment of the HyHEL-10 antibody, and development of biophysical methods of determining thermodynamic driving forces and effects of mutation on affinity and specificity.

Construction of recombinant expression vectors for hen egg lysozyme in *E. coli*, and more recently in yeast will allow the production of directed mutant forms of the lysozyme antigen to test the contributions of specific residues to antibody-antigen binding affinity and selectivity. Our original pKK223-3 based system for expression of hen lysozyme in *E. coli*, included the strong hybrid *tac* promoter followed by the pUC8 multiple cloning site, which is positioned in the vector such that genes cloned into the Eco RI or Sma I sites can utilize the adjacent ribosomal binding site provided by the vector. Strong transcription terminators immediately follow the multiple cloning site to stabilize the plasmid. A chicken cDNA fragment obtained from G. Schutz was used as the source for the lysozyme gene.

The protein was well expressed from this *lac*-based vector, but cells expressing lysozyme grew very slowly. While lysozyme should not attack the cell wall when expressed cytoplasmically, it is apparently toxic by non-specific mechanisms (electrostatic interactions of the highly cationic protein with nucleic acids?). Strains with enhanced *lac* repressor activity showed improved growth, and we also placed our constructs under the tighter control of the lambda P_L promoter, using a vector designed for this purpose (plasmid pP_L, Pharmacia). In the final analysis, however, neither of these stategies was successful in alleviating the problem of toxicity.

We have also obtained an *S. cerevisiae* expression vector for HEL as the generous gift of I. Kumagai (University of Tokyo), and the *leu2* host strain AH22 from the laboratory of G. Fink (MIT) and have obtained secretion of active lysozyme. Since

the submission of the third year progress report we have also used the PCR to easily construct a new *S. cerevisiae* expression vector based on plasmids obtained from S. Elliot (AMGEN, Inc.). This phagemid-based system offers tremendous convenience in mutagenesis and sequencing, and should allow increased productivity in generation of HEL mutant forms.

We have recently completed the construction of an expression vector for antibodies of the HyHEL series as chimeric Fab fragments in *E. coli*. We have used PCR to selectively amplify the sequences coding for the variable regions of the HyHEL-10 antibody heavy and light chains from cDNA clones provided by our NIH collaborators, and to introduce unique restriction sites at the ends of each sequence. Using vectors developed by S. Ward at the MRC Laboratory of Molecular Biology, Cambridge, we have genetically fused these sequences to human CH1 and Cκ sequences, respectively, and to leader sequences from *pelB* of *Erwinia herbicola*. Both sequences are downstream of a common promoter, leading to formation of a dicistronic message followed by simultaneous secretion of the two Fab chains into the periplasm. This strategy has repeatedly been successfully used for expression of correctly-folded Fab fragments, and we now have immunochemical evidence of the formation of the correct chimeric HyHEL-10 Fab molecules in E. coli supernatant. We look forward to using *E. coli* expression in antibody engineering studies.

We have completed the extensive preparations necessary for obtaining antibodies in the quantities and purities needed, and for reliable measurements of the enthalpy of antibody-antigen association by titration microcalorimetry, and are well along on the characterization of the HyHEL-5/HEL system. Results indicate that the association is *enthalpically* driven, in contrast to the conventional picture of hydrophobically-driven association based on entropic driving forces. The enthalpy of association is also rather strongly temperature-dependent. We anticipate submitting a manuscript on these results, the first calorimetric characterization of a structurally-defined antibody/protein complex, in the near future. As HyHEL-5/HEL affinity involves a particularly large contribution from electrostatic interactions, it will be of great interest to see if the thermodynamic driving force is the same for the less-electrostatic HyHEL-10/HEL complex, and for HyHEL-5 association with lysozyme variants incapable of forming major salt links. We are actively pursuing these questions.

As expected, titration microcalorimetry does not provide useful information on the affinities of association of our (very tight) wild-type antibody/HEL complexes (it can be used to measure the affinities of complexes of lower affinity). We have acquired a high-sensitivity SPEX 212 fluorometer for use in the measurement of antibody-antigen affinities in solution (as opposed to the less-reliable values available from solid phase assays). This instrument will also serve as the basis of a program of measurement of antibody-antigen association rates, in conjunction with Brownian dynamics simulation of diffusional encounter in solution.

SIGNIFICANCE: The three theoretical subprojects are intended to provide better methods for predicting the structures of antibody binding sites, the effects of salt and dielectric environment on biomolecular associations, and the free energy of antigen-antibody interactions in water. The experiments test the structural and thermodynamic determinants of antibody affinity and binding kinetics. Comparison of the theoretical and experimental results allows a detailed insight into molecular recognition in antibody-antigen binding.

WORK PLAN: The period of ONR support for this project under the Accelerated Research Initiative in Molecular Recognition is expiring, but we will continue to pursue work in this area, building on the results obtained with ONR support. For the first theoretical subproject, we will attempt to obtain crystals of the anti-insulin antibody suitable for testing the theoretical model by means of X-ray diffraction structure determination. For the second theoretical subproject, work will continue on the optimization of the various types of simulation, and on the experimental testing of the electrostatics/Brownian dynamics calculations. The third theoretical subproject has been successfully completed, and the results will be submitted for publication shortly (McCammon, Pomes, Willson). In the experimental area, we will continue to test the contributions of critical contacts to antibody affinity and specificity, with the added flexibility of access to antibody mutants. We will also devote an increasing amount of effort to measuring the kinetics of electrostatically-mediated diffusional diffusional encounter, and the effects on kinetics of changes in protein structure and the solvent environment.

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